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(54) Title: PROCESS FOR PREPARING CYCLOSPORIN A

(57) Abstract

According to the present invention, a process for the preparation of cyclosporin A comprising the steps of adding lower alkanol solvent to a culture fluid of a mutant of *Tolypocladium inflatum* and extracting the mixture; transferring the extract to a halogenated lower hydrocarbon solvent; if required, the resultant solution being treated with activated charcoal; and the solution being column chromatographed on silica gel is provided. The process of the present invention is advantageous from the aspect of process economics and environmental protection because the culture fluid is directly extracted without filtration, drying procedure is omitted and the chromatography process is simplified to a single process using silica gel.

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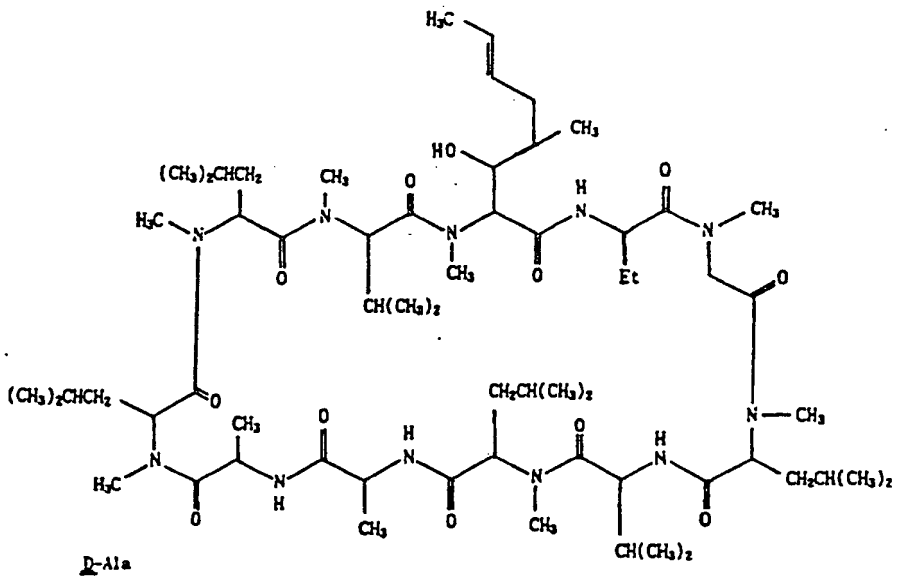
TITLE OF THE INVENTION

PROCESS FOR PREPARING CYCLOSPORIN A

TECHNICAL FIELD

The present invention relates to a novel process for the preparation of cyclosporin A represented by following structural formula(I).



Cyclosporin A represented by the above structural formula is a cyclic peptide of molecular weight 1202, molecular formula $C_{62}H_{111}N_{11}O_{12}$, consisting of eleven(11) amino acids, and obtained from a culture fluid of Tolypocladium inflatum, a strain producing cyclosporin A. There are twenty five(25) derivatives of cyclosporin A dependent upon the types of amino acids [Traber R., HELVETICA ACTA, 70,

13(1987)].

Chemical name of cyclosporin A is cyclo[((E) - (2S,3R,4R) - 3 - hydroxy - 4 - methyl - 2 - (methylamino) - octenoyl) - L - 2 - aminobutyryl - N-methyl - glycyl - N-methyl - L - leucyl - L - valyl - N-methyl - L - leucyl - L - alanyl - O - alanyl - N-methyl - L - leucyl - N-methyl - L - leucyl - N-methyl - L - valyl]. Showing very strong immunosuppressive effect, cyclosporin A has been used for suppressing rejection symptoms and treating auto-immune diseases. Also, it is known to have antifungal, insecticidal and anti-inflammatory effect [Borel J.F., Prog. Allergy, 38, 9(1986)].

BACKGROUND ART

As prior arts concerning the process for preparing cyclosporin A, methods using fermentation by Cylindrocarpon lucidum and Tolypocladium inflatum are disclosed in GB Patent 1,491,509 and US Patent 4,117,118, and US Patent 4,215,199. Also, various methods to recover cyclosporin A from the culture fluid are disclosed in these patents.

In the above-mentioned patents, a process comprising the steps of extracting mycellium isolated from the culture fluid with a solvent, and then recovering the product by successively using chromatography media such as sephadex, aluminum oxide and silica gel has been used as a process for recovering cyclosporin A from the culture fluid. In accordance with these processes, solvent is added to mycellium as isolated solid obtained by filtering the culture fluid; the mixture is extracted by solvent by means of centrifugal extracter; the resultant extract is pretreated with water or hexane or the like; and then the

product is purified through chromatography media such as sephadex, aluminum oxide and silica gel, etc. to obtain cyclosporin A. However, the procedure is complicated because two or three types of chromatography media including sephadex should be used after extracting the mycellium as isolated solid obtained by filtering the culture fluid with solvent. Besides, the processes may cause problems of process economics and environmental pollution because each eluting solvent shoud be used for each chromatography procedure.

Thus, in order to overcome the complexity of the processes and lighten the economic burden, various methods for purifying cyclosporin A have been studied.

For example, according to the teachings of US Patent 5,256,547 and European Patent 057,968 A1, mycellium as isolated solid obtained by filtering the culture fluid is dried and then extracted with a solvent to obtain crude cyclosporin A; the product is pretreated without washing with water or the like, and purified by means of chromatography using aluminum oxide and silica gel avoiding the use of expensive sephadex to obtain cyclosporin A. In addition, according to the teachings of US Patent 5,156,960, the culture fluid is extracted by directly adding solvent, the extract is transferred to a solvent having higher solubility and dried over a drying agent such as anhydrous magnesium sulfate, and the resultant product is purified by chromatography through sephadex and silica gel etc. to obtain cyclosporin A.

Although the art described in US Patent 5,256,547 has advantages in that content of pigment has been reduced by virtue of the solvent extraction and the process has been simplified in the course of solvent pretreatment and chromatography, it also has disadvantage in that an extra

process for drying the mycellium should be performed. On the other hand, the art described in US Patent 5,156,960 has an advantages in that solvent extraction is carried out directly from the culture fluid by simply adding a solvent instead of solvent extraction from mycellium separated from the culture fluid after filtration, and the processes through chromatography media are performed without pretreatment with solvent. However, there should be an additional process for drying over expensive anhydrous magnesium sulfate because a significant amount of moisture is admixed in the transferring solvent owing to the curtailment of the process, and two chromatography media of sephadex and silica gel are used so that it still involves problems of complexity and economic disadvantage so as to be industrialized.

DISCLOSURE OF INVENTION

The present inventors have intensively studied to solve the problems of the prior art, and completed a novel economic process for preparing cyclosporin A comprising the steps of solvent extraction by directly adding a solvent to the culture fluid instead of the solvent extraction of mycellium isolated by filtering the culture fluid; and single chromatography using silica gel media without drying process using anhydrous magnesium sulfate.

The present invention relates to a process for the preparation of cyclosporin A comprising the steps of adding alkanol solvent to a culture fluid of a mutant of Tolypocladium inflatum and extracting the mixture; transferring the extract to a lower chlorohydrocarbon solvent having higher solubility; treating the solution

with activated charcoal; and the resultant product being column chromatographed on silica gel.

During the extraction process of the culture fluid, alkanol solvent is used. The amount of the alkanol solvent to be added is desirably about twice by volume of the culture fluid, and extraction is preferrably carried out for about 30 minutes with stirring.

As lower chlorohydrocarbon solvent to which the product is transferred, methylene chloride, ethylene chloride, chloroform, or the like may be used. Among these solvents, methylene chloride is preferably used. Prior to the treatment through chromatography media, it is preferred to treat the product with activated charcoal in order to remove the colored impurity.

In the process according to the present invention, drying process which have been the big problem of the prior art to be industrialized is omitted, and the moisture content of the extracted solution is far lowered by using a nonpolar lower chlorohydrocarbon solvent.

In addition, the purity of the cyclosporin A was improved by virtue of the choice of lower chlorohydrocarbon as a transferring solvent and the treatment with activated charcoal, and a significant amount of solvent is saved because of the simplification of the chromatography procedure so that lessen the environmental burden.

BEST MODE FOR CARRYING OUT THE INVENTION

Now, the present invention is further described by referring to the following examples. However, the present invention should not be understood to be limited to the examples.

Example 1

Three(3) liters of methanol were added to 1.5 liters of culture fluid containing cyclosporin A(7,115 μg/ml), and the mixture was filtered to collect the extracted cyclosporin A. The extract obtained was washed with 2 liters of hexane, and the product was further extracted by adding 2 liters of methylene chloride. Thirty grams of activated charcoal were added to the mixture in order to remove colored impurity. After filtration, the mixture was evaporated to dryness.

The product obtained was dissolved in 50 ml of ethyl acetate and the solution was loaded to the top of the silica gel column. After eluting the product by ethyl acetate, the combined fractions (3,000 ml) containing the desired product were evaporated to dryness. Three mililiters of ethyl ether were added to dissolve the product, and added 150 ml of methoxymethane to the mixture. The product precipitated was isolated to obtain cyclosporin A (6.4 g, content:98.5-100.0%).

Example 2

Three(3) liters of methanol were added to 1.5 liters of culture fluid containing cyclosporin A (6,757 μg/ml), and the mixture was filtered to collect the extracted cyclosporin A. After concentrating the extract to have a volume of 1.1 liter, the concentrate was washed with 1.1 liter of methoxymethane, and the resultant product was extracted twice with 1.1 liter of methylene chloride each.

The product was treated with activated charcoal and chromatographed on silica gel in accordance with Example 1 to obtain cyclosporin A(7.09 g, content:98.5-100.0%).

Example 3

The same procedure as Example 1 was repeated but washing the methanol extract with 2 liters of methoxymethane, to obtain cyclosporin A (content:98.5-100.0%).

WHAT IS CLAIMED IS :

1. A process for the preparation of cyclosporin A comprising the steps of adding lower alkanol solvent to a culture fluid of a mutant of Tolypocladium inflatum and extracting the mixture; transferring the extract to a halogenated lower hydrocarbon solvent; and the resultant product being column chromatographed on silica gel.

2. A process according to claim 1, where the transferred halogenated hydrocarbon solution is treated by activated charcoal.

3. A process according to claim 1 or 2, where the halogenated lower hydrocarbon solvent is selected from a group consisting of methylene chloride, ethylene chloride and chloroform.

4. A process according to claim 3, where the halogenated lower hydrocarbon solvent is methylene chloride.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 95/00095

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
DD A5 298276	13-02-92	keine - none - rien	
US A 5256547	26-10-93	AT E 127528	15-09-95
		DE C0 59106428	12-10-95
		EP A1 507968	14-10-92
		EP B1 507968	06-09-95
		HU A2 61605	28-01-93
		HU A0 911160	28-10-91
		HU B 211026	28-09-95

Form PCT/ISA/210 (patent family annex) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 95/00095

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 P 21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 P 21/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DD 298 276 A5 (INSTITUT FÜR MIKROBIOLOGIE UND EXPERIMENTELLE THERAPIE) 13 February 1992 (13.02.92), claims 1,4,5.	1
A	US 5 256 547 A (RUDAT et al.) 26 October 1993 (26.10.93), abstract.	1

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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